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TIM3⁺FOXP3⁺ regulatory T cells are tissue-specific promoters of T-cell dysfunction in cancer

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Abbreviations: NSCLC, non-small cell lung cancer; TIL, tumor-infiltrating lymphocytes; TIM3, T-cell immunoglobulin mucin 3; Treg, regulatory T cell

T-cell immunoglobulin mucin 3 (TIM3) is an inhibitory molecule that has emerged as a key regulator of dysfunctional or exhausted CD8⁺ T cells arising in chronic diseases such as cancer. In addition to exhausted CD8⁺ T cells, highly suppressive regulatory T cells (Tregs) represent a significant barrier against the induction of antitumor immunity. We have found that the majority of intratumoral FOXP3⁺ Tregs express TIM3. TIM3⁺ Tregs co-express PD-1, are highly suppressive and comprise a specialized subset of tissue Tregs that are rarely observed in the peripheral tissues or blood of tumor-bearing mice. The co-blockade of the TIM3 and PD-1 signaling pathways *in vivo* results in the downregulation of molecules associated with TIM3⁺ Treg suppressor functions. This suggests that the potent clinical efficacy of co-blocking TIM3 and PD-1 signal transduction cascades likely stems from the reversal of T-cell exhaustion combined with the inhibition of regulatory T-cell function in tumor tissues. Interestingly, we find that TIM3⁺ Tregs accumulate in the tumor tissue prior to the appearance of exhausted CD8⁺ T cells, and that the depletion of Tregs at this stage interferes with the development of the exhausted phenotype by CD8⁺ T cells. Collectively, our data indicate that TIM3 marks highly suppressive tissue-resident Tregs that play an important role in shaping the antitumor immune response *in situ*, increasing the value of TIM3-targeting therapeutic strategies against cancer.

Introduction

T-cell immunoglobulin mucin 3 (TIM3) was originally identified as a molecule selectively expressed on terminally differentiated interferon γ (IFN γ)-producing Type 1 CD4⁺ helper T (T_H1) and Type 1 CD8⁺ cytotoxic T (T_C1) cells.¹ Upon binding to galectin-9, a soluble s-type lectin, TIM3 triggers cell death.² Thus, TIM3 operates as an inhibitory molecule that ensures the proper termination of T_H1/T_C1 driven inflammation. Accordingly, the blockade of TIM3 signaling has been shown to increase IFN γ secretion and to increase the susceptibility of mice to develop multiple autoimmune conditions in which IFN γ -secreting cells play a major role and to exacerbate the severity of the disease.^{1,3–5} Similarly, blocking the TIM3 signal transduction cascade by antibodies or RNA interference increases the secretion of IFN γ by activated human T cells.^{4,5}

Recent exciting findings from us⁶ and others^{7–11} have further characterized TIM3 as a key regulator of the dysfunctional or exhausted CD8⁺ T-cell phenotype that arises in the course of chronic diseases, including chronic viral infections and cancer, in

both humans and experimental models. Exhausted T cells fail to proliferate and mediate effector functions (cytotoxicity and cytokine production) in response to antigen stimulation and hence pose a significant barrier to the induction of productive antiviral and antitumor immunity.

We were the first to show that the most exhausted population of CD8⁺ T cells in cancer is marked by the expression of both TIM3 and the inhibitory receptor PD-1 (TIM3⁺PD-1⁺), the latter of which can also be found on cells that retain effector functions (TIM3⁺PD-1⁻).⁶ We also demonstrated that the co-blockade of the TIM3 and PD-1 signaling pathways reproducibly induces tumor regression, while the inhibition of PD-1 signals alone provides inconsistent and/or less robust antitumor effects.^{6,12,13} Accordingly, the co-blockade of TIM3 and PD-1 signals is more effective than the inhibition of the PD-1 signal transduction cascade alone at enhancing effector T-cell responses in both tumor-infiltrating lymphocytes (TILs) and peripheral T cells in tumor-bearing mice⁶ as well as in T cells obtained from advanced melanoma patients.⁹ These observations clearly indicate that TIM3 plays an important role in regulating the exhausted phenotype of CD8⁺ T cells. Still,

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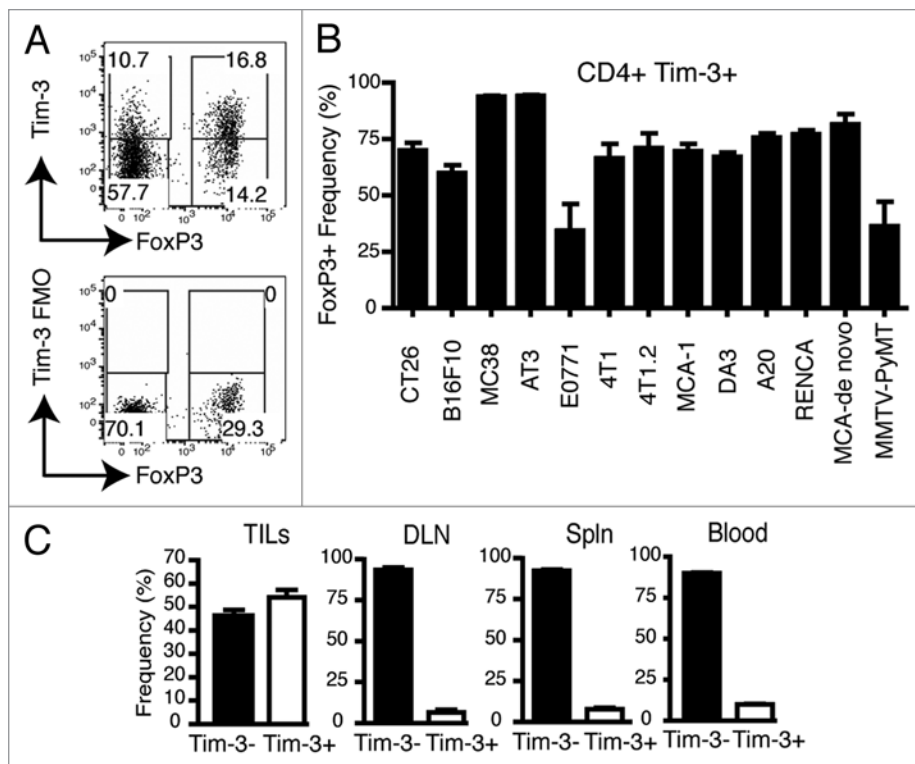


Figure 1. TIM3 expression on FOXP3⁻ and FOXP3⁺CD4⁺ tumor-infiltrating lymphocytes. (A) Representative co-expression of TIM3 and FOXP3 on CD4⁺ lymphocytes infiltrating CT26 colon carcinomas. (B) Frequency of FOXP3⁺ cells within TIM3⁺CD4⁺ cells infiltrating the indicated solid tumors. (C) Frequency of TIM3⁻ and TIM3⁺ cells within FOXP3⁺CD4⁺ T cells found in neoplastic lesions, draining lymph nodes (DLNs), spleen and blood of mice bearing subcutaneous CT26 carcinomas (n = 5). Error bars indicate SEM.

whether TIM3 plays any role in additional cancer-elicited mechanisms of T-cell suppression remains unknown.

We have previously demonstrated that CD4⁺ TILs express TIM3,^{6,12} yet the significance of TIM3⁺CD4⁺ TILs in antitumor immune responses was not investigated in details. Here, we report that TIM3 identifies intratumoral Tregs that exert robust immunosuppressive functions and have a major role in driving the development of dysfunctional/exhausted CD8⁺ T cells.

Results

TIM3⁺FOXP3⁺ CD4⁺ T cells are specifically present within the tumor microenvironment. We have previously observed that TIM3 is expressed by CD4⁺ TILs.^{6,12} However, we did not determine whether these CD4⁺TIM3⁺ TILs exhibit a FOXP3⁻ effector and/or a FOXP3⁺ regulatory phenotype. As CD4⁺FOXP3⁺ Tregs are known to expand during tumor progression^{14,15} and to play a significant role in suppressing antitumor immunity,^{16–19} we examined the expression of TIM3 on FOXP3⁺ vs. FOXP3⁻CD4⁺ TILs infiltrating CT26 cell-derived colon carcinomas. To our surprise, we found that more than 50% of FOXP3⁺ Tregs were TIM3⁺ (Fig. 1A–C). In sharp contrast, less than 10% of CD4⁺FOXP3⁻ TILs expressed TIM3 (Fig. S1A). Indeed, the overwhelming majority of CD4⁺TIM3⁺ TILs infiltrating multiple distinct solid

tumors were FOXP3⁺ (Fig. 1B), indicating that the predominance of TIM3⁺ Tregs among TILs is a common feature across cancers of different histological origin. Interestingly, when we examined the presence of TIM3⁺ Tregs in the blood, spleen and tumor-draining lymph nodes of tumor-bearing mice, we found that TIM3⁺FOXP3⁺ Tregs are present at high frequencies only in tumor tissues (Fig. 1C; Fig. S1B), suggesting that TIM3⁺ Tregs may comprise a specialized subset of Tregs that specifically accumulates within the tumor microenvironment.

Since we have previously observed that TIM3 and PD-1 are co-expressed on exhausted CD8⁺ TILs,⁶ we examined the expression of PD-1 on TIM3⁺ Tregs and found that PD-1 is co-expressed with TIM3 on Tregs (Fig. S1C). However, unlike TIM3, PD-1 is not differentially expressed on CD4⁺FOXP3⁺ vs. CD4⁺FOXP3⁻ cells (Fig. S1D). Thus, TIM3 marks both exhausted CD8⁺ T cells and Tregs in the tumor microenvironment, while PD-1 does not. Collectively, our data indicate that TIM3⁺ Tregs uniquely accumulate within tumor tissues and may comprise a specialized subset of tissue-resident Tregs that plays an important role in shaping

antitumor immune responses in situ.

TIM3⁺ Tregs are more immunosuppressive than TIM3⁻ Tregs. Given their predominance in the tumor microenvironment, we next characterized the immunosuppressive function of TIM3⁺ Tregs. To this aim, we isolated TIM3⁺ and TIM3⁻FOXP3⁺ CD4⁺ T cells infiltrating CT26 tumors growing in FOXP3-GFP knock-in mice (which express the green-fluorescence protein in FOXP3⁺ cells only) and compared their suppressor functions ex vivo, in a suppression assay. We found that TIM3⁺ Tregs are approximately 2-fold more immunosuppressive than TIM3⁻ Tregs (Fig. 2A). We then examined the expression of the immunosuppressive cytokine interleukin (IL)-10 and of cytotoxic effector molecules in TIM3⁺ and TIM3⁻ Tregs ex vivo. We found that the TIM3⁺ Treg population contains a 2-fold higher frequency of IL-10-producing cells than its TIM3⁻ counterpart (Fig. 2B). This was also true for TIM3⁺ Tregs infiltrating B16 melanomas (Fig. S1E). In addition, we found that TIM3⁺ Tregs express more perforin, granzyme A and granzyme G than TIM3⁻ Tregs (Fig. 2C). Collectively, these data indicate that TIM3⁺ Tregs represent activated tumor tissue Tregs that exhibit superior immunosuppressive activity as compared with TIM3⁻ Tregs.

Modulation of TIM3⁺ Treg suppressor function by TIM3/PD-1 pathway co-blockade. The combined blockade of the TIM3 and PD-1 signaling pathways is highly effective in

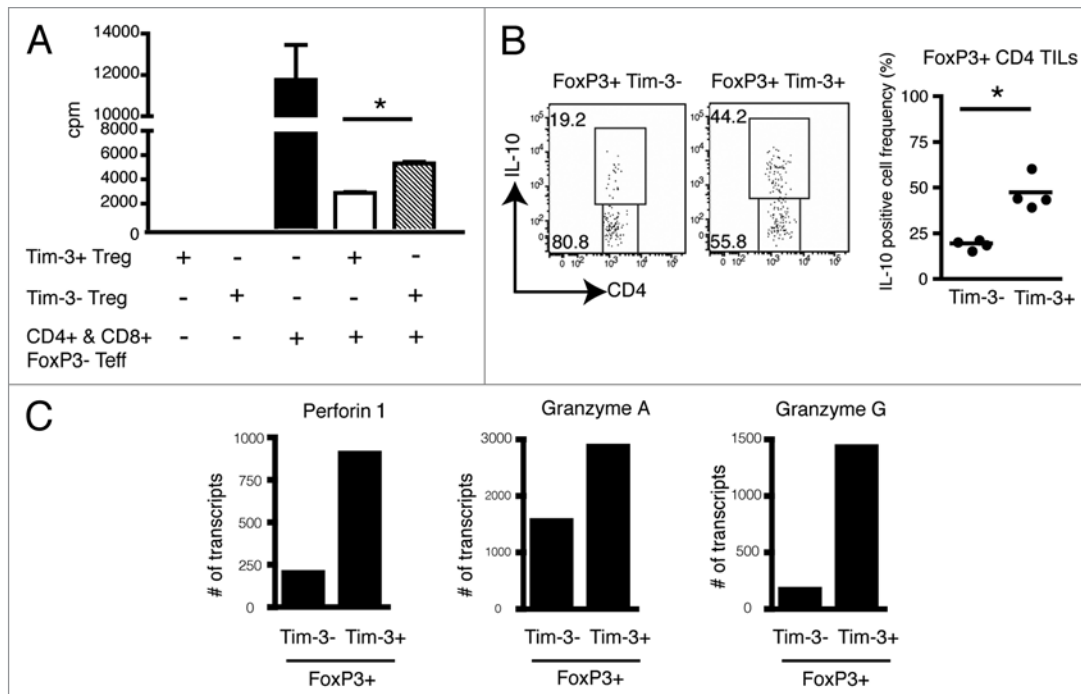


Figure 2. Functional phenotype of TIM3⁺ and TIM3⁻ FOXP3⁺ regulatory T cells. **(A)** In vitro suppression assay. FOXP3⁻CD4⁺ and CD8⁺ splenocytes from tumor-naïve mice were cultured alone or together with TIM3⁺ or TIM3⁻ FOXP3⁺CD4⁺ lymphocytes infiltrating CT26 carcinomas at a 1:4 regulatory T-cell (Treg): effector T-cell (Teff) ratio. Proliferation was determined by [³H]-thymidine incorporation. Data are representative of two independent experiments. *p = 0.0031 (Student's t-test). **(B)** Left panel, representative interleukin-10 (IL-10) intracytoplasmic staining in TIM3⁺ and TIM3⁻ FOXP3⁺CD4⁺ infiltrating CT26 carcinomas. Right panel, frequency of IL-10⁺ cells among TIM3⁺ vs. TIM3⁻ FOXP3⁺ CD4⁺ tumor-infiltrating lymphocytes (n = 4). *p = 0.0011 (Student's t-test). This experiment has been repeated twice, yielding similar results. **(C)** Expression of perforin 1, granzyme A and granzyme G in TIM3⁺ vs. TIM3⁻ FOXP3⁺CD4⁺ TILs. Gene expression was analyzed by NanoString nCounter. Data are representative of one out of two independent assays.

controlling tumor growth and restoring the function to exhausted CD8⁺ T cells, whereas the inhibition of PD-1-transduced signals alone results in inconsistent and less robust effects.^{6,9,12} As TIM3 marks both tumor-infiltrating Tregs and exhausted CD8⁺ TILs while PD-1 does not (Fig. S1D),⁶ the remarkable clinical efficacy of the TIM3/PD-1 co-blockade might stem from combined effects on TIM3⁺ Tregs and TIM3⁺ exhausted CD8⁺ TILs. We therefore profiled TIM3⁺ Tregs from tumor-bearing mice subjected to the combined blockade of the TIM3 and PD-1 signaling pathways (with specific monoclonal antibodies) using the multiplex NanoString nCounter technology. We found that several molecules associated with Treg suppressor function are downregulated in TIM3⁺ Tregs upon the administration of blocking antibodies in vivo (Fig. 3). Perforin was downregulated by 5-fold, suggesting that TIM3⁺ Tregs exert reduced cytotoxic functions upon TIM3/PD-1 co-blockade. The expression of the inhibitory molecules PD-1 and LAG-3, which have been previously implicated in supporting Treg function,^{20,21} was also reduced by 2.5- and 5-fold, respectively.

Multiple signaling pathways that impact on T-cell activation and trafficking were also downregulated in this setting. The Type II IL-1 receptor (IL-1R2), which indirectly decreases T-cell activation as it functions as a decoy receptor for IL-1 α and IL-1 β , was downregulated by 2-fold. The regulator of G-protein signaling 16 (RGS16), which controls the activity of several chemokine receptors including CCR5, was downregulated by 11-fold.

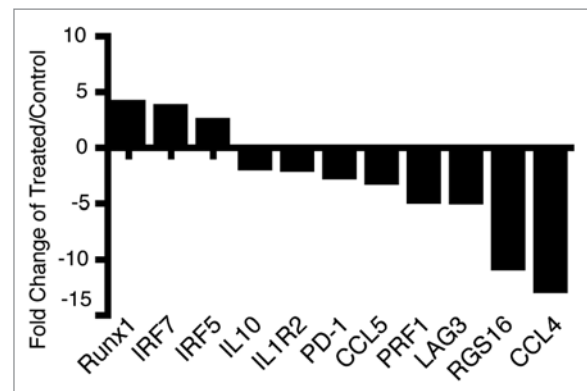


Figure 3. Downregulation of regulatory T cell (Treg) effector molecules in TIM3⁺ Tregs upon in vivo TIM3/PD-1 co-blockade. Mice bearing CT26 carcinomas (n = 10) were treated with 100 μ g anti-TIM3 antibodies (on day 0, 2, 4), 200 μ g anti-PD-L1 antibodies (on day 0, 3, 6, 9, 12) or appropriate isotype-matched control antibodies, followed by the isolation of TIM3⁺FOXP3⁺CD4⁺ tumor-infiltrating lymphocytes (TILs). Gene expression was then analyzed by NanoString nCounter. Data are represented as fold changes of treated vs. control animals.

Interestingly, two ligands of CCR5 (CCL5 and CCL4) were also downregulated, by 3- and 12-fold, respectively. CCR5 is known to play a role in the recruitment of Tregs to tumors,²² while CCL4 has been shown to inhibit T-cell activation by interfering

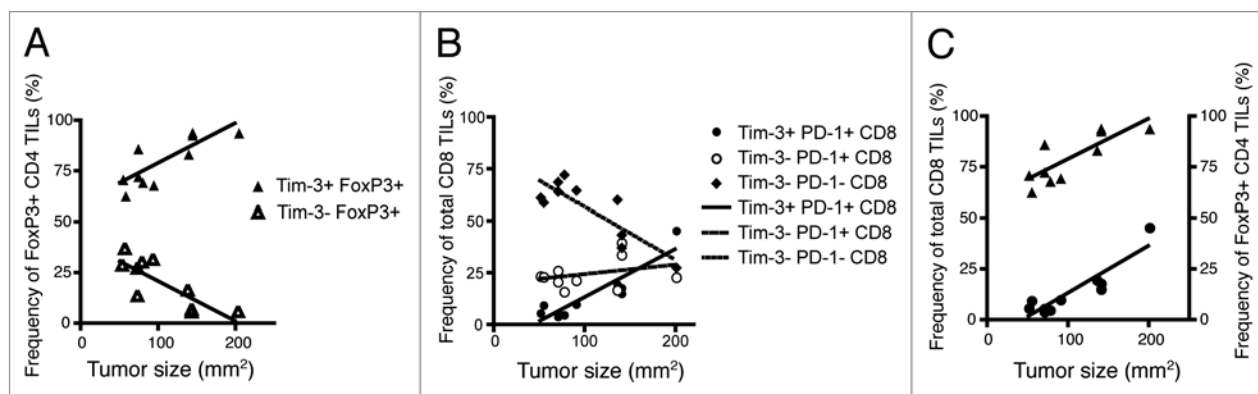


Figure 4. Intratumoral accumulation of TIM3-expressing regulatory T cells and exhausted CD8⁺ T cells during tumor progression. **(A and B)** Tumor-infiltrating lymphocytes were harvested from B16F10 melanoma-bearing mice at different time points post-implantation and subjected to flow cytometry. **(A)** Frequency of TIM3⁺ and TIM3⁻ cells among FOXP3⁺CD4⁺ TILs. The Pearson R values for TIM3⁺ and TIM3⁻ regulatory cells (Tregs) are 0.808 and -0.8085, respectively. **(B)** Frequency of CD8⁺ TILs subpopulations expressing TIM3 and PD-1. For CD8⁺TIM3⁺PD-1⁺ TILs, R = 0.9107; for CD8⁺TIM3⁺PD-1⁻ TILs, R = 0.3022, for CD8⁺TIM3⁻PD-1⁻ TILs, R = -0.8433.

with T-cell receptor signaling.²³ Thus, the co-administration of anti-TIM3 and anti-PD-L1 antibodies reduces the immunosuppressive functions of TIM3⁺ Tregs and may alter their homing to and/or retention within neoplastic lesions.

A few genes were upregulated following the co-blockade of TIM3 and PD-1 in vivo, including *Runx1*, which regulates IFN γ and IL-2 signaling in Tregs,²⁴ and two members of the IFN-regulatory factor transcription factor family, i.e., *Irf5* and *Irf7*. These latter observations suggest a previously unrecognized role for IRFs in the regulation of Treg function.

Among the factors modulated by the co-blockade of TIM3 and PD-1 in vivo, IL-10 is of special interest. We have shown that TIM3⁺ Tregs produce high amounts of IL-10 (Fig. 2; Fig S1E), and this cytokine has previously been implicated in the development of T-cell exhaustion during chronic viral infection (reviewed in ref. 25). Taken together, these observations raised the possibility that TIM3⁺ Tregs may have a role in promoting T-cell dysfunction in the tumor microenvironment.

Relationship between TIM3⁺ Tregs, exhausted CD8⁺ T cells and tumor growth. To address whether TIM3⁺ Tregs may have a role in promoting T-cell exhaustion, we performed a kinetic analysis of intratumoral TIM3⁺ Tregs and exhausted CD8⁺ TIM3⁺PD-1⁺ T cells during tumor progression. Interestingly, we found that TIM3⁺ Tregs are the dominant Treg population at all time points in both the B16 melanoma and CT26 colon carcinoma models (Fig. 4A; Fig. S2A). Moreover, TIM3⁺ Tregs clearly accumulate in parallel to the growth of B16 melanoma.

We next examined the CD8⁺ T-cell compartment. It is generally recognized that the presence of exhausted CD8⁺ T cells strongly correlates with tumor growth. As expected, we found that TIM3⁺PD-1⁺ CD8⁺ cells, which represent the most exhausted CD8⁺ TILs, are present at a very low frequency within small B16 melanoma and CT26 colon carcinoma but accumulate steadily along with tumor growth (Fig. 4B; Fig. S2B). Overall, our kinetic studies show that TIM3⁺ Tregs dominate the Treg population and that the presence of TIM3⁺ Tregs precedes the appearance of exhausted CD8⁺ TIM3⁺PD-1⁺ TILs. Based on

these data, we hypothesized that TIM3⁺ Tregs are specialized tissue-resident Tregs that accumulate early in the tumor microenvironment where they promote the neoplastic growth by supporting the development of dysfunctional CD8⁺ T cells.

The depletion of FOXP3⁺ Tregs dramatically alters the development of exhausted CD8⁺ TILs and augments effector T-cell functions. To test the hypothesis that TIM3⁺ Tregs may play a critical role in the acquisition of a dysfunctional phenotype by CD8⁺ T cells, we examined the consequences of a transient FOXP3⁺ T-cell depletion during the early phases of B16 melanoma growth, when TIM3⁺ Tregs constitute approximately 75% of tumor-infiltrating Tregs and CD8⁺TIM3⁺PD-1⁺ exhausted TILs have not yet accumulated (Fig. 4). Our kinetic study suggested that this occurs around day 8–12 post-implantation, when the surface of neoplastic lesions is roughly 80–100 mm². We therefore administered the diphtheria toxin (DT) to mice stably expressing the DT receptor under the control of the FOXP3 promoter (FOXP3-DTR mice) 8 or 9 days after the implant of B16 cells (Fig. 5A). Our working model predicted that the depletion of Tregs would result in a decrease in CD8⁺TIM3⁺PD-1⁺ T cells. Surprisingly, we found that the frequency of CD8⁺TIM3⁺PD-1⁺ T cells, which exhibit an exhausted phenotype in normal tumor-bearing mice, increased upon the depletion of Tregs (Fig. 5B and C). However, when we examined their functions, we found that CD8⁺TIM3⁺PD-1⁺ TILs produce significant amounts of IL-2, tumor necrosis factor α (TNF α), and IFN γ and therefore no longer exhibit an exhausted phenotype (Fig. 5D).

In addition to the dramatic alteration in the effector phenotype of CD8⁺TIM3⁺PD-1⁺ TILs, the depletion of Tregs promoted the emergence of a population of CD8⁺TIM3⁺PD-1⁻ T cells, which is generally not observed in tumor-bearing mice (Fig. 5B and E). These CD8⁺TIM3⁺PD-1⁻ cells produce both IFN γ and TNF α , indicating that they operate as effector cells (Fig. 5F). In contrast to the overall increase in TIM3⁺ cells, both the CD8⁺TIM3⁻PD-1⁻ and CD8⁺TIM3⁻PD-1⁺ TIL subpopulations are decreased in Treg-depleted mice (Fig. 5B; Fig. S3).

The examination of the CD4⁺ cell compartment revealed drastic alterations affecting the CD4⁺FOXP3⁻ effector T-cell population. In particular, following the depletion of Tregs we observed the emergence of a significant CD4⁺FOXP3⁻TIM3⁺ cell population. This cell subset was present at a very low frequency in control tumor-bearing mice (Fig. 5B and G). These CD4⁺FOXP3⁻TIM3⁺ cells are mostly IFN γ producers, de facto accounting for the overall increase in IFN γ -producing cells among CD4⁺FOXP3⁻ TILs in Treg-depleted mice (Fig. 5H). Collectively, these observations indicate that FOXP3⁺ Tregs, whose tumor-infiltrating subset mostly display a TIM3⁺ phenotype, support the development of exhausted CD8⁺ T cells and limit the expansion of both CD4⁺ and CD8⁺ effector T cells that secrete pro-inflammatory cytokines (IFN γ and TNF α) within the tumor microenvironment.

Synergy between TIM3 blockade and FOXP3⁺ Treg depletion. Our data indicate that CD4⁺ and CD8⁺ IFN γ -producing effector T cells emerge in tumor-bearing mice upon the depletion of Tregs (Fig. 5). These cells are not exhausted but rather express TIM3 as a consequence of their activation and differentiation toward an IFN γ -producing effector phenotype. If this were indeed the case, TIM3⁺ effector T cells should be subjected to regulation by TIM3-transduced signals and hence the blockade of TIM3 in Treg-depleted mice would provide additional therapeutic benefits as compared with those provided by the depletion of Tregs. As a matter of fact, we found that while a single dose of DT to CT26 colon carcinoma-bearing FOXP3-DTR mice delays tumor growth only transiently, the administration of TIM3-blocking antibodies to Treg-depleted mice results in significant and sustained tumor regression (Fig. 6A). The synergistic effects of Treg depletion plus TIM3 blockade were also observed in both MC38 carcinoma and B16 melanoma models (Fig. S4A). Lastly, we observed

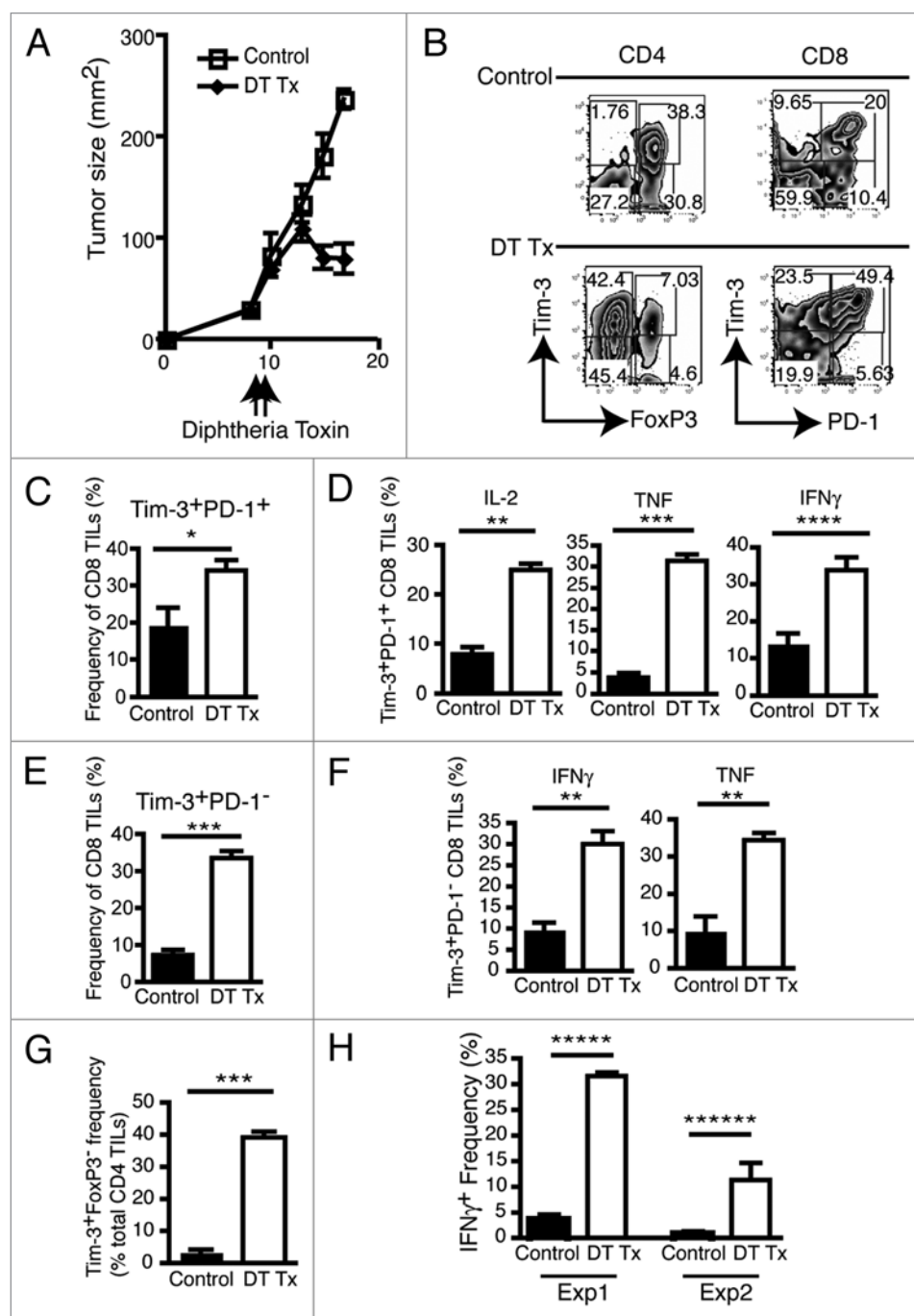


Figure 5. FOXP3⁺ T-cell depletion dramatically alters the accumulation of exhausted CD8⁺ cells and the effector T-cell phenotype in the tumor microenvironment. (A–H) B16F10 melanoma cells were implanted in mice expressing the diphtheria toxin (DT) receptor (DTR) under the control of the FOXP3 promoter (FOXP3-DTR mice). DT or PBS was then injected on days 8/9 post tumor-implantation. (A) Effect of FOXP3⁺ T-cell depletion on tumor growth (mean \pm SEM, $n = 10$). (B) Representative FACS data showing the expression of TIM3 on CD4⁺ and CD8⁺ tumor-infiltrating lymphocytes (TILs) as purified from control and DT-treated tumor-bearing mice on day 14. (C–H) Frequency of CD8⁺TIM3⁺PD-1⁺ TILs [(C), $n = 10$]; TIM3⁺PD-1⁺ TILs producing interleukin-2 (IL-2), tumor necrosis factor α (TNF α), or interferon γ (IFN γ) [(D), $n = 8$]; CD8⁺TIM3⁺PD-1⁺ TILs [(E), $n = 10$]; TIM3⁺PD-1⁺ TILs producing TNF α and IFN γ [(F), $n = 8$]; CD4⁺TIM3⁺FOXP3⁺ TILs [(G), $n = 10$]; and IFN γ -producing cells within CD4⁺FOXP3⁺ TILs in control vs. DT-treated tumor-bearing mice. All data except those in (H) are pooled from three independent experiments. Error bars indicate SEM * $p = 0.0165$, ** $p = 0.0001$, *** $p < 0.0001$, **** $p = 0.0047$ (Student's t -test). In (H), data pooled from two independent experiments are shown, with $n = 4$ for experiment 1 and $n = 3$ for experiment 2. ***** $p = 0.0173$, ***** $p = 0.0437$ (Student's t -test).

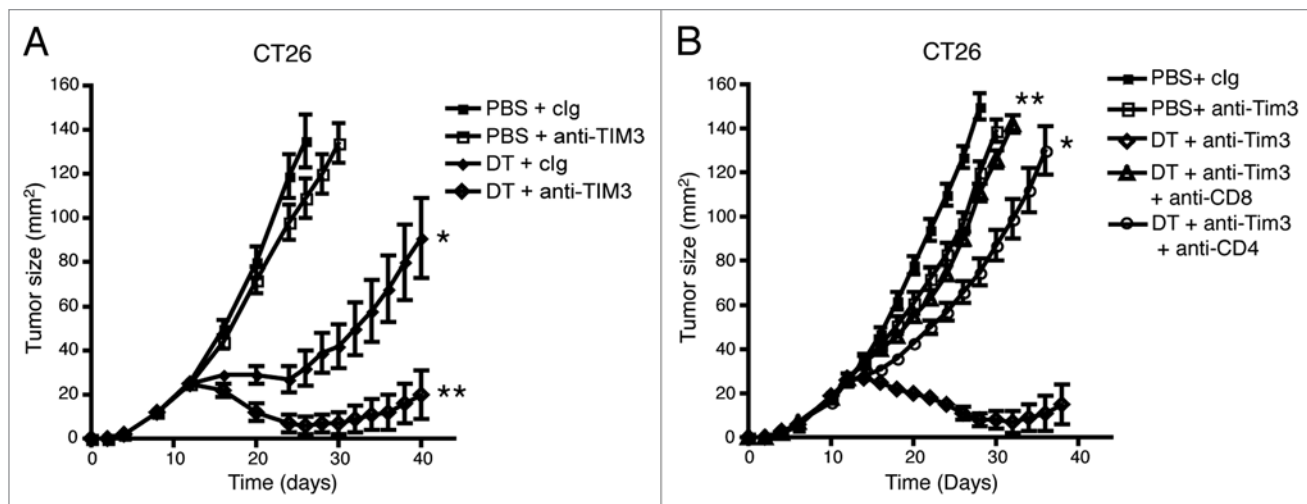


Figure 6. Synergistic effects of TIM3 blockade and FOXP3⁺ regulatory T-cell depletion. (**A and B**) CT26 colon carcinoma cells (1×10^5) were implanted in mice expressing the diphtheria toxin (DT) receptor (DTR) under the control of the FOXP3 promoter (FOXP3-DTR mice) and either 0.5 μ g DT or PBS was injected on day 12 post-tumor implantation. The anti-TIM3 antibody RMT3-23 (250 μ g) or an appropriate isotype-matched control antibody (clg) was given every 4 d from day 12 to day 24 post-tumor implantation. (**A**) Effects of FOXP3⁺ T-cell depletion and anti-TIM3 antibodies on tumor growth. *PBS/clg vs. DT/clg, $p = 0.0079$ on day 24; **DT/clg vs. DT/RMT3-23, $p = 0.0593$ on day 24, $p = 0.0362$ on day 26, $p = 0.0273$ on day 28, $p = 0.0362$ on day 40. (**B**) Mice were treated as in (**A**) in combination with either an anti-CD8 β or an anti-CD4 antibody. *DT/RMT3-23 vs. DT/RMT3-23/anti-CD4, $p = 0.0079$ on day 24, $p = 0.0119$ on day 28, $p = 0.0079$ on day 32, $p = 0.0119$ on day 36; **DT/RMT3-23 vs. DT/RMT3-23/anti-CD8, $p = 0.0079$ on day 24, $p = 0.0119$ on day 28, $p = 0.0079$ on day 32. Tumor sizes are represented as mean \pm SEM. Both experiments have been repeated twice. Statistical differences were calculated by Mann-Whitney analyses at the indicated time points.

that this synergy is completely abolished when either CD4⁺ or CD8⁺ T cells are depleted (Fig. 6B; Fig. S4B), indicating that both the CD4⁺ and CD8⁺ effector T cells that emerge upon Treg depletion play a major role in mediating tumor regression in this experimental setting.

Discussion

In addition to restoring the effector activity of exhausted T cells, interfering with regulatory T-cell function is highly desirable in clinical settings. Our data indicate that TIM3 marks intratumoral Tregs and that the co-blockade of the TIM3 and PD-1 signaling pathways results in the downregulation of effector molecules in TIM3⁺ Tregs. Taken together with the fact that TIM3 marks intratumoral exhausted CD8⁺ T cells and that the simultaneous inhibition of TIM3- and PD-1-transduced signals restores the function to these cells,⁶ these observations position TIM3 as an ideal candidate for cancer immunotherapy. Our data suggest that TIM3 inhibition early during tumor development would mainly target tumor-infiltrating TIM3⁺ Tregs, de facto interfering with their immunosuppressive function and inhibiting the development of CD8⁺ T-cell exhaustion. TIM3 blockade at later time points would additionally target exhausted CD8⁺ T cells and restore their effector functions. Unlike the global inhibition of FOXP3⁺ cells, anti-TIM3 antibodies would selectively target intratumoral Tregs, lessening the potential for undesirable autoimmune side effects. In this sense, the clinical relevance of TIM3⁺ Tregs as an immunotherapeutic target is supported by the recent observation of CD4⁺TIM3⁺FOXP3⁺ cells in patients affected by non-small cell lung cancer (NSCLC)²⁶

and infected by the hepatitis C virus.²⁷ Interestingly, in concordance with our findings, CD4⁺TIM3⁺FOXP3⁺ cells can be found among TILs but not in the peripheral blood of NSCLC patients, and the presence of TIM3⁺ Tregs correlates with poor clinical prognosis.

TIM3⁺ Tregs have recently been detected within allografted tissue during graft rejection.²⁸ There are some similarities between the TIM3⁺ Tregs that arise during such an acute inflammatory response and those that develop during cancer-associated chronic inflammation, but also notable differences. In both cases, TIM3⁺ Tregs exert more robust immunosuppressive functions and express higher levels of effector molecules (such as IL-10) as compared with their TIM3⁻ counterparts. However, TIM3⁺ Tregs constitute less than half of the Treg population that develop in response to allograft and appear to be short-lived. It has indeed been proposed that in this setting TIM3 operates as a death-inducing molecule, eliminating highly immunosuppressive Tregs and allowing for the re-establishment of immune homeostasis. In stark contrast, TIM3⁺ Tregs clearly constitute the predominant Treg population throughout all phases of tumor progression. The fact that TIM3 does not eliminate these cells likely represents yet another example of deregulation that arises in chronic diseases, similar to the persistence of TIM3⁺ exhausted CD8⁺ T cells in cancer.

A deeper understanding of the mechanisms whereby TIM3 affects the function of Tregs and promotes T-cell exhaustion will prove critical as TIM3 moves forward as a candidate target for anticancer immunotherapy. Our NanoString profiling data show that several molecules that support the function of Tregs (IL-10, perforin, PD-1, LAG-3) are downregulated upon the co-blockade

of TIM3 and PD-1 signaling pathways. As IL-10 has been implicated in T-cell exhaustion in chronic viral infection,²⁵ it is possible that this cytokine mediates one mechanism by which TIM3⁺ Tregs drive the development of CD8⁺ T-cell exhaustion in cancer. PD-1 and LAG-3 have been shown to support Treg function, yet they are also expressed on exhausted CD8⁺ T cells to operate as inhibitory receptors. All these observations point to an ongoing conundrum in the field: how do molecules that inhibit effector T cells support Treg function? Our data support the role of such molecules in Treg suppressor function and open the possibility that the signaling pathways that are elicited in Tregs by these factors play a prominent role in promoting T-cell exhaustion.

We found that several molecules affecting T-cell trafficking to and/or retention within the tumor tissue (e.g., CCL4, CCL5, RGS16) are downregulated following the co-inhibition of the TIM3 and PD-1 signaling pathways. The expression of these molecules on TIM3⁺ Tregs could account, at least in part, for the high frequency of TIM3⁺ Tregs in the tumor microenvironment. In addition, the expression of galectin-9 on neoplastic cells including B16 melanoma and CT26 colon carcinoma cells,^{6,12} may also influence the retention of TIM3⁺ Tregs in the tumor tissue. Whether galectin-9/TIM3 interactions play a role in the promotion of T-cell exhaustion by TIM3⁺ Tregs remains to be determined.

We also found that a few genes are upregulated in response to the co-blockade of TIM3- and PD-1-transduced signals in vivo, including *Runx1*, *Irf5* and *Irf7*. RUNX1 normally activates IL-2 and IFN γ signaling, but in Tregs appears to mediate a reverse effect owing to its association with FOXP3.²⁴ As a possibility, the upregulation of Runx1 in TIM3⁺ Tregs may increase the amount of free Runx1, thereby favoring the activation of IL-2/IFN γ signaling. IRF5 and IRF7 are mediators of Type 1 IFN signaling that have been primarily characterized in macrophages. Our findings suggest a role for these factors in the regulation of Treg suppressor functions.

The prophylactic depletion of Tregs results in the eradication of some tumors, such as leukemia, sarcoma and myeloma.²⁹ However, their therapeutic depletion (once neoplastic lesions are established) is often scarcely effective, even when combined with other immunotherapeutic approaches.³⁰ In contrast, we have shown that the depletion of Tregs in mice bearing established tumors and the blockade of TIM3 signaling exert synergistic anti-neoplastic effects, even against poorly immunogenic B16 melanomas. Interestingly, the antitumor activity of TIM3 blockade coupled to Treg depletion, like that of TIM3 inhibition alone,¹² was completely abolished upon the depletion of either CD4⁺ or CD8⁺ T cells. TIM3 represents an important target on both CD4⁺ and CD8⁺ effector T cells and either subset alone is capable of significant antitumor activity. Our findings provide the first rationale for combining TIM3-blocking antibodies with Treg-targeted therapies that are already approved for use in cancer patients.¹⁷

The relative importance of TIM3 in Tregs vs. exhausted T cells for antitumor immunity remains unclear. In order to conclusively address this issue, the generation of a *Tim3^{flax/flax}* mouse in which the conditional deletion of *Tim3* in Tregs vs. CD8⁺ T cells can be achieved is required. Nevertheless, our data clearly

indicate that TIM3 marks highly suppressive Tregs that are present uniquely within the tumor microenvironment, where they play a role in shaping antitumor immune responses. Thus, our observations increase the potential value of TIM3 as a target for anticancer immunotherapy.

Materials and Methods

Mice. Six- to 12-week old female mice were used in all studies. BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory. FOXP3-DTR KI C57BL/6 mice were kindly provided by Dr. Alexander Y. Rudensky. These mice were backcrossed > 7 times onto BALB/c mice and it was determined that this strain accepted BALB/c tumors. IL-10-Thy1.1 C57BL/6 mice were kindly provided by Dr. Casey T. Weaver. FOXP3-GFP KI C57BL/6 mice were generated in Dr. Kuchroo's laboratory and backcrossed onto the BALB/c background. DEREK mice on a C57BL/6 background were kindly provided by Dr. Tim Sparwasser.¹⁸ All experiments were approved and conducted according to the guidelines set forth by the Harvard Medical Area Standing Committee on Animals and The Peter MacCallum Animal Experimentation Ethics Committee.

Antibodies and reagents. Anti-TIM3 (RMT3-23, kindly provided by Hideo Yagita, Juntendo University) and isotype control (rat IgG2a) antibodies were purchased from BioXCell. The following fluorochrome-conjugated antibodies were purchased from Biolegend: anti-CD8a (53-6.7), anti-CD4 (RM4-5), anti-CD45.2 (104), anti-PD-1 (RMP1-30), anti-FOXP3 (MF-14 and FJK-16s), anti-IFN γ (XMG1.2), anti-TNF α (MP6-XT22) and anti-IL-10 (JES5-16E3). Anti-TIM3 (8B.2C12 and RMT3-23) and anti-IL-2 (JES6-5H4) antibodies were purchased from eBioscience. The anti-TIM3 5D12 antibody was generated in V.K.K.'s laboratory and conjugated to phycoerythrin (PE) and allophycocyanin (APC) by BioLegend.

Tumor experiments. CT26, B16F10, MC38, A20 and RENCA cells were purchased from ATCC. AT3, EO771, 4T1, 4T1.2 and DA3 mammary tumors were derived and maintained as previously described.³¹ BALB/c MCA-1 cells were derived from a sarcoma induced in BALB/c mice by methylcholanthrene (MCA). CT26 and MC38 (1×10^6), B6F10 and AT3 (5×10^5), A20 (2×10^5), BALB/c MCA-1 and DA3 (1×10^5), 4T1 and 4T1.2 (5×10^4) were implanted s.c. in the flanks of mice. EO771 (5×10^5) and RENCA (2×10^5) were implanted orthotopically. Tumor size was measured in two dimensions by a common caliper and is reported here as the product of two perpendicular diameters. Tumors arising in wild-type C57BL/6 mice treated with MCA (MCA de novo) or C57BL/6 MMTV-PyMT mice were also harvested and assessed for TILs. For the depletion of FOXP3⁺ cells, FOXP3-DTR mice were implanted with B16F10 cells on day 0 and treated with 50 μ g/kg DT i.p. on days 8 and 9. For the studies examining the synergy between anti-TIM3 antibodies and Treg depletion, FOXP3-DTR mice were treated with DT on day 12, and 250 μ g anti-TIM3 RMT3-23 or isotype control antibodies were given on days 12, 16, 20 and 24. In some groups of mice, 100 μ g anti-CD4 (GK1.5) or anti-CD8 β (53.5.8) were given i.p. on days 11, 12, 19 and 26.

Lymphocyte isolation. For phenotypic and functional assays, TILs were isolated as previously described.^{6,12} Briefly, tumors were dissected, dissociated in a gentle MACS dissociator (Miltenyi Biotec) and then digested with collagenase D prior to separation on a discontinuous Percoll gradient (GE Healthcare). Splenocytes and lymphocytes from ipsilateral inguinal lymph nodes (draining lymph nodes) were also isolated and characterized in some experiments.

Flow cytometry. Single-cell suspensions were stained for surface markers and—after fixation—for intracellular FOXP3 using an anti-FOXP3 staining kit (eBioscience). 7-Aminoactinomycin D (7AAD) was employed to exclude dead cells from the analysis. Cytoplasmic cytokine staining was conducted as previously described.⁶ All data were collected on an LSR II (BD) or Loader Canto (BD) fluorometer and analyzed with FlowJo software (Tree Star, Inc.).

In vitro suppression assays. TIM3⁺FOXP3⁺ and TIM3-FOXP3⁺ CD4⁺ cells were sorted from TILs along with splenic FOXP3-CD4⁺ cells and CD8⁺ cells to be used as effector T cells. TIM3⁺ or TIM3-FOXP3⁺ Tregs were then cultured with 1.5×10^4 of CD4⁺/CD8⁺ effector T cells at 1:4 ratio in the presence of 1 μ g/ml soluble anti-CD3 antibody and 7.5×10^4 irradiated splenocytes obtained from a non-tumor bearing mouse. After 48 h, cells were pulsed with [³H]-thymidine and harvested 18 h later. [³H]-Thymidine incorporation was measured on a micro β counter.

Gene expression analyses by NanoString. CT26 tumor cells were implanted in FOXP3-GFP KI mice. For the analysis of TIM3⁺ and TIM3-FOXP3⁺ TILs ex vivo, cells were isolated by cell sorting on day 16 post-tumor implantation and immediately lysed in RLT buffer (Qiagen). For the analysis of TIM3⁺ Treg upon TIM3/PD-1 co-blockade in vivo, CT26 carcinoma-bearing mice were treated with anti-TIM3 plus anti-PD-L1 or isotype control antibodies as previously described.⁶ On day 16 post-tumor implantation, CD4⁺TIM3⁺FOXP3⁺ TILs were isolated by cell sorting and immediately lysed in RLT buffer. Cell lysates were hybridized with a custom made CodeSet. Barcodes were counted (1150 fields of view per sample) on an nCounter Digital Analyzer following the manufacturer's protocol (NanoString

Technologies, Inc.). Data were first normalized with respect to the geometric mean of the positive control spike counts (provided by the manufacturer) then relative to four reference genes (*Actb*, *Gapdh*, *Hprt* and *Tubb5*). A background correction was done by subtracting the mean + 2 SDs of eight negative control counts (provided by the manufacturer) and eliminating conditions that were < 1.

Statistical analyses. Statistical analyses were performed using the Prism 5.0 software (GraphPad Software, Inc.). Student's t-tests and linear regression analyses were applied when appropriate. Statistical differences between tumor sizes of different mice groups were determined by Mann-Whitney tests. p values < 0.05 were considered as statistically significant.

Disclosure of Potential Conflicts of Interest

ACA is a paid member of the Scientific Advisory Board of CoStim Pharmaceuticals, which has interests in cancer immunotherapy.

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Supplemental Materials

Supplemental materials may be found here: <http://www.landesbioscience.com/journals/oncoimmunology/article/23849/>

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